Novel therapeutic targets for the treatment of asthma

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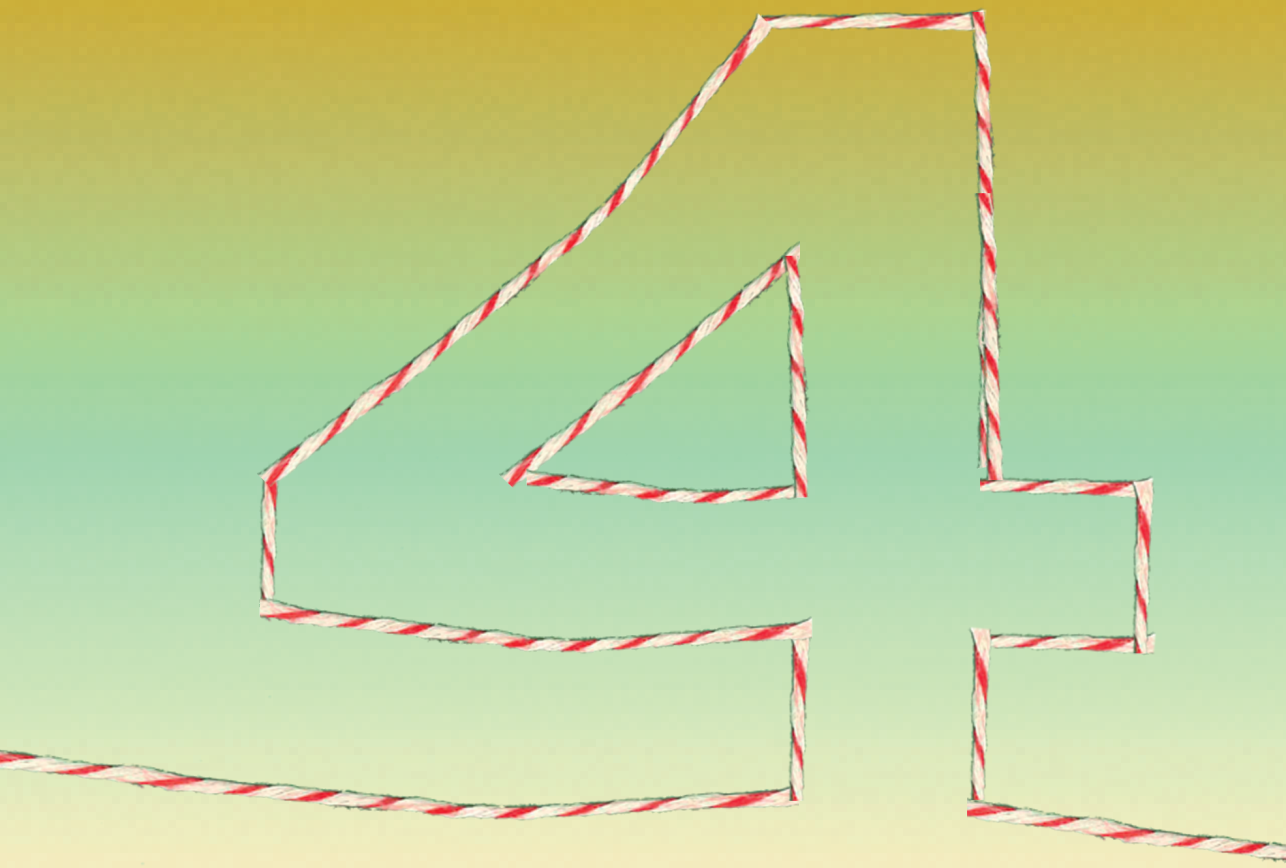

Arginase 2 regulates mast cell proliferation and differentiation via ornithine metabolism

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Abstract

Arginase is a metalloenzyme responsible for the catalytic conversion of L-arginine into L-ornithine and urea. Arginase 1 has previously been linked to the pathophysiology of asthma. The role of arginase 2 is less clear as both enhancing and inhibitory effects of arginase 2 on asthma have been suggested. Using precision-cut lung slices of mice deficient in arginase 2, we found a protective effect of this gene on mast cell activation, as compound 48/80-induced airway narrowing was slightly increased in *Arg2*^{KO} mice compared to wild-type animals, without an effect on methacholine-induced airway narrowing. Further experiments using the human mast cell line LUVA showed that arginase 2 inhibition inhibits mast cell division, reduces mast cell size, yet increases the gene expression of FcεR1 and enzymes involved in mast cell-mediator production (e.g. ALOX15, PTGS2, TPH2) indicating an inhibitory role for arginase 2 in mast cell differentiation. Intracellular L-ornithine levels were decreased after arginase inhibition and inhibition of L-ornithine decarboxylase (ODC) led to similar effects on cell division, size and gene expression as inhibition of arginase 2. Combined treatment with spermidine was able to reverse the effects of ODC inhibition on mast cell division. Together this indicates that the protective role of arginase 2 on mast cell dependent airway narrowing, is the result of the promotion of mast cell proliferation rather than differentiation, which is mediated via the regulation of L-ornithine metabolism.

Introduction

Allergic asthma is a chronic disease of the airways, affecting over 300 million people world-wide. It is characterized by airway hyperresponsiveness, obstruction, inflammation and remodeling (1). Mast cells play an important role in allergic asthma (2). Following allergen sensitization, mast cells express immunoglobulin (Ig)E-molecules specific to the allergen. On subsequent encounters, the allergen will bind to the IgE/high-affinity IgE receptor FcεRI complexes on the surface of mast cells leading to receptor cross-linking and mast cell activation (3, 4). Upon activation mast cells will degranulate and release pre-made and stored proinflammatory mediators, such as histamine, from their granules. In addition, *de novo* synthesized mediators, such as leukotrienes, prostaglandins and cytokines, are released. Within minutes after allergen encounter, mast cell mediators promote smooth muscle contraction, vascular permeability and mucous secretion (2, 5, 6). Moreover, chemokines released by mast cells contribute to the recruitment of inflammatory cells (mainly eosinophils), which promote the late phase asthmatic response. This late phase response is associated with swelling of the bronchial wall, smooth muscle contraction, mucus production, as well as increased non-specific airway hyperresponsiveness (2).

The metalloenzyme arginase is able to attenuate the production of nitric oxide (NO) by nitric oxide synthases (NOS) (7-9). Furthermore, the arginase enzymes are responsible for

the catalytic conversion of L-arginine to L-ornithine and urea. Subsequent metabolism of L-ornithine by ornithine decarboxylase (ODC) leads to the formation of the polyamines putrescine, spermidine and spermine (10). In mammals, two arginase isoenzymes can be identified: arginase 1 and arginase 2. Arginase 1 is located in the cell cytosol and is highly expressed in hepatic cells, whereas arginase 2 is found in the mitochondria of cells (11). Both isoenzymes can be found throughout the body, including in the lung. Data obtained from animal studies have shown a role for increased arginase activity or expression in allergic asthma (7, 9, 12-15). Furthermore, genome-wide association studies found several single nucleotide polymorphisms (SNPs) of both the arginase 1 (*ARG1*) and arginase 2 (*ARG2*) gene that correlated with asthma development, severity and treatment success (16-18). The role of arginase in allergic asthma can be substantiated by findings of elevated arginase levels in serum and airways of asthmatic patients compared to healthy controls, and the association between disease severity, arginase expression in bronchial brushings, serum arginase activity, plasma L-arginine and L-arginine metabolite levels (13, 14, 19, 20). Accordingly, arginase is viewed as a potential therapeutic target for the treatment of allergic asthma (21).

Studies in animal models of asthma have shown that inhibition of arginase 1 may indeed be beneficial, and have shown its involvement in airway hyperresponsiveness and airway inflammation (12, 22-24). However, less is known of the role of arginase 2 in asthma pathology. In a guinea pig model of chronic asthma, increased arginase 2 expression and activity was associated with a modulation of lung parenchyma specific hyperresponsiveness (25). In contrast, high enzyme activity of arginase 2 has also been linked to decreased asthma severity and suppression of Th2-driven airway inflammation in both human bronchial epithelial cells and a mouse model of asthma (26). Furthermore, arginase 2 is associated with asthma severity, and *ARG2* SNP variants associated with lower arginase activity are associated with more severe asthma (27, 28). The role of arginase 2 is thus not fully established as both enhancing and inhibitory effects of arginase 2 have been suggested and common *ARG2* SNPs for asthma can be linked to both increased and decreased *ARG2* gene expression (29).

The IPF Cell Atlas indicates arginase 2 is the main arginase expressed by mast cells in the human lung (30). However, its functional role in mast cells is still unknown. This study set out to gain more insight into the role of arginase 2 in mast cells. To achieve this, precision-cut lung slices of mice deficient in *Arg2* were used to study the role of arginase 2 in mast cell-induced airway narrowing. Furthermore, using the human mast cell line LUVA, predominantly expressing arginase 2, we were able to find a protective role of arginase 2 on mast cell proliferation by inhibiting differentiation, which appeared to be mediated via the regulation of L-ornithine metabolism.

Materials and methods

Animals

Homozygous female Arg2tm1Weo mice (The Jackson Laboratory stock #020286)(31) were crossed with male C57BL/6J mice to set up colonies of heterozygous mice for this study. Heterozygous F1 mice were crossed to generate homozygous *Arg2*^{KO} offspring and wild-type littermate controls, which were used for experiments. All animals were sensitized to ovalbumin on day 0, 14 and 21 by intraperitoneal injection of 200 µl of 10 µg ovalbumin in 1.5 mg $Al(OH)_3$ in saline (32). Animals were housed under a 12 h light/dark cycle with controlled humidity and room temperature at 24 ± 1 °C. Water and food were provided *ad libitum*. All experiments were performed according to the national guidelines and upon approval of the experimental procedures by the local Animal Care and Use committee of the University of Groningen (application number: AVD1050020184845)

Allergen challenge and tissue collection

For arginase 1 and 2 gene expression experiments, ovalbumin-challenged mice were used. In brief, the ovalbumin sensitized mice were challenged with 1% ovalbumin aerosols in PBS, or saline as control, for 20 minutes, twice weekly on consecutive days for four weeks. Challenges were performed in a Perspex exposure chamber (9l) using a De Vilbiss nebulizer (type 646; De Vilbiss, Somerset, PA) driven by an airflow of 40 L/min, resulting in an aerosol output of 0.33 ml/min, as described previously (32). One day after the last challenge, animals were sacrificed by subcutaneous injection of ketamine and the lungs were collected and snap-frozen for mRNA analysis. The snap-frozen and stored mouse lung tissue originates from a previous study (33).

Precision-cut lung slices

Ovalbumin-sensitized wild-type and *Arg2*^{KO}-mice were used to prepare precision-cut lung slices. Lung slices were prepared as described previously (34, 35). In short, mice were euthanized by subcutaneous injection of ketamine (40 mg/kg, Alfasan, Woerden, The Netherlands) and dexdomitor (0.5 mg/kg, Orion Pharma, Mechelen, Belgium). Subsequently, a small incision was made in the trachea to insert a cannula. Through the cannula, the lungs were inflated with a low melting-point agarose solution (1.5% final concentration (Gerbu Biotechnik GmbH, Wieblingen, Germany) in $CaCl_2$ (0.9 mM), $MgSO_4$ (0.4 mM), KCl (2.7 mM), NaCl (58.2 mM), NaH_2PO_4 (0.6 mM), glucose (8.4 mM), $NaHCO_3$ (13 mM), Hepes (12.6 mM), sodium pyruvate (0.5 mM), glutamine (1 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100), pH = 7.2). To solidify the agarose, the lungs were covered with ice for 20 minutes after which the they were harvested. The lungs were divided in separate lobes and cut into 250 µm thick lung slices in cold medium composed of $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH_2PO_4 (1.2 mM), glucose (16.7 mM), $NaHCO_3$ (26.1 mM), HEPES (25.2 mM), pH = 7.2, using a tissue slicer (Leica VT 1000 S Vibrating blade microtome,

Leica Biosystems B.V., Amsterdam, The Netherlands). The lung slices were washed for four times, placed in DMEM supplemented with sodium pyruvate (1 mM), MEM non-essential amino acid mixture (1:100; Gibco® by Life Technologies), gentamycin (45 µg/ml; Gibco® by Life Technologies), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (1.5 µg/ml; Gibco® by Life Technologies) and incubated in a humidified atmosphere under 5% CO_2/95% air at 37 °C. Lung slices were used for airway narrowing studies or placed in a 24 wells plate, using three to four slices per well, and treated with ovalbumin (1 mg/ml) for 24h to use for mRNA analysis.

Airway narrowing studies

Lung slices of wild-type and *Arg2*^{KO}-mice were used to perform airway narrowing studies, as described previously (34, 35). Lung slices were placed in 1 ml medium and fixed using a nylon mesh and metal washer. Dose response curves for metacholine (10^{-9}-10^{-3} M) and compound 48/80 (1-1000 µg/ml) were established at 37 °C. Time-lapse images of the lung slices (1 frame per 2 s) were captured using a microscope (Eclipse, TS100; Nikon). The airway luminal area was quantified using image acquisition software (NIS-elements; Nikon) and expressed as percentage of basal area, as done previously (34, 35).

Cell culture and stimulation

LUVA human mast cells (Kerafast, Inc., Boston, MA) were cultured in StemPro 34 serum-free medium supplemented with StemPro® 34 nutrient supplement, 50 units/ml penicillin plus 50 µg/ml streptomycin (5 ml), 1.5 µg/ml amphotericin B (3 ml) and 200 mM L-glutamine (5 ml). LUVA cells were seeded in a 6 wells plate with a cell density of 5 x 10^5 cells/ml in 1 ml medium, for rtPCR. For cell counting experiments, cells were seeded in 24 wells plates in a density of 5 x 10^4 cells/ml in 500 µl medium. For flow cytometry, cells were seeded in 24 wells plates in a cell density of 1 x 10^5 cells/ml in 500 µl medium. For cell size measurements, cells were seeded in 24 wells plates in a cell density of 5 x 10^5 cells/ml in 500 µl medium. For L-arginine metabolism measurement, cells were seeded in 6 wells plates in a cell density of 5 x 10^5 cells/ml in 1 ml medium. Cells were treated with the arginase inhibitor 2(*S*)-Amino-6-boronohexanoic acid (ABH; 10 µM; synthesized as described in (36)), the ODC inhibitor α-DFMO (5 mM; Sigma-Aldrich Chemie N.V., Zwijndrecht, Netherlands) and/or spermidine (100 µM; Sigma-Aldrich Chemie N.V., Zwijndrecht, Netherlands) for up to 72h.

mRNA isolation and PCR analysis

Total mRNA of lung slices was isolated using the Maxwell 16 instrument and corresponding Maxwell 16 LEV simply RNA tissue kit (Promega, Madison, USA) for automated purification, according to the manufacturer's instructions. While Trizol RNA extraction (TRI Reagent Solution, Applied Biosystems, Landsmeer, Netherlands) was used for LUVA cells, according to the manufacturer's instructions. cDNA was synthesized from equal amounts of RNA using Reverse Transcriptase System (Promega, Madison,

WI, USA), and the following protocol: 10 min 25 °C, 45 min 42 °C, 5 min 99 °C. rtPCR was performed with SYBR Green (Roche Diagnostics, Almere, Netherlands) and the following protocol including a final step to generate the melting curve: 2 min 95 °C, 10 min 95 °C, 45× (30 s 95 °C, 30 s 60 °C, 30 s 72 °C), 30 s 95 °C, 30 s 55 °C, 30 s 95 °C. The rtPCR was performed in an Eco Illumina (Illumina, Eindhoven, Netherlands). For analysis, the LinReg software was used to calculate N0-values which were normalized to N0 of *the housekeeping genes* 18s (lung slice) or 18s and GAPHD (LUVA) as an internal control. Primer sets used to analyze gene expression in lung slices and LUVA cells are shown in Table 1 and Table 2, respectively.

Table 1: Primers used for rtPCR of mouse precision cut lung slices.

Gene:	Forward primer	Reverse primer:
S18	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
ARG1	CAGCACTGAGGAAAGCTGGT	CAGACCGTGGGTTCTTCACA
ARG2	TAGGGTAATCCCCTCCCTGC	AGCAAGCCAGCTTCTCGAAT
IL13	CAGCCTCCCCGATACCAAAA	TCCTCATTAGAAGGGGCCGT

Table 2: Primers used for rtPCR of LUVA human mast cells.

Gene:	Forward primer	Reverse primer:
S18	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
GAPDH	CCAGCAAGAGCACAAGAGGA	GAGATTCAGTGTGGTGGGGG
ARG1	GGAGACCACAGTTTGGCAAT	CCACTTGTGGTTGTCAGTGG
ARG2	TGCATCCTTGAACTGTCAGC	ACAAGCTGCTGCTTTCCATT
PTGS2	AGCCCATTGAACCTGGACTG	ACCCAATCAGCGTTTCTCGT
HPGDS	AGGCTGTAGAGACAAGGAAAGG	AGTGCCACTGTATTGCTGCC
ALOX15	GAATACCTTGGGCCACTGCT	GTCAGAGATACTGGTCGCCG
ALOX5	CCTCGTCACTCGGTAGCATC	GCAGAAAGGGCCACTGGTAT
TPH1	TAGAAGTATGTCCACGGGCCT	GGACGGATGGAAAACCCAGT
TPH2	CCCTACACGCAGAGCATTGA	TTCCACAACTCACCGACTGG
HDC	GGAGCCCTGTGAATACCGTG	CCCAGGCATGATGACTCGTT
TPSAB1	TGGCCAAGCCATTAGAGACC	CACAGTGAGGAGTAGTGCGG
CMA1	AAGCCCCCTGCTGTCTTTAC	TGCAGTTGACAATCTGGGTCT
FCER1a	ATAAAAGCTCCGCGTGAGAA	GAAATGTGACCTGCTGCTGA
KIT	TCATGGTCGGATCACAAAGA	AGGGGCTGCTTCCTAAAGAG

Cell counting

In order to quantify cell division, pretreated and untreated LUVA cells were monitored by automated cell counter system with an interval of 24 hours for 5 days. In order to do this, 20 µl of cells was suspended in 10 ml isotonic CasyTon buffer in combination with ZAP-OLGOBIN II lytic reagent and measured with the Casy TT cell counting device (Roche Innovatis, Bielefield, Germany). The cell counting was performed three times within one measurement of which the average was used.

Cell size measurement

Cell size was measured 30 minutes, 24, 48 and 72 hours after treatment. The cell samples were diluted with medium to a concentration of 5 x 10^5 cells/ml if needed. Cytospin-preparations were made using a Shandon cytospin 3 cytrocentrifuge and stained with Toluidine blue for better visualization. Per cytospin, the size of 20 cells were determined at 5 different places in the coupe using a microscope (Eclipse, TS100; Nikon) at 20x magnification and the image acquisition software (NIS-elements; Nikon).

Flow cytometry

To study cell viability AnnexinV/PI (Fisher Scientific, Landsmeer, The Netherlands) staining was performed as described in (37) after 72 hours of treatment. Flow cytometric analysis was performed using the Cytoflex (Beckman Coulter, Woerden, the Netherlands). Cells staining negative for both AnnexinV and propidium iodide (PI) were counted as viable cells. Data were collected from 15 000 cells per condition and analysed using Kaluza Analysis Software (Beckman Coulter, Woerden, the Netherlands).

Amino acid LC-MS/MS measurements

After 24 hours of treatment cells were centrifuged at 900 rpm for 5 minutes. The cell pellets were stored at -80°C until analysis. For urea cycle amino acids analysis, cells were samples were thawed on the fridge and thereafter 25 µl of cell supernatant were crashed in an Eppendorf tube with 225 µl of 0.2 % formic acid in isopropanol containing the internal standard (1 µmol/L of N4-Arginine). After vortexing for 20 seconds, samples were left at room temperature for 10 minutes and then centrifuged at 10,000 g for 15 minutes. Finally, samples were transferred to an LC-MS vial for their analysis

Urea cycle amino acid analyses were performed on an ACQUITY UPLC System from Waters Corporation (Milford, MA, USA) coupled to a Waters Xevo® TQ-S triple quadrupole system equipped with an Electrospray Ion Source as previously described (Madhi et al, 2018). Separation was carried out on a SeQuant® ZIC®-HILIC (100 X 2.1 mm, 3.5 µm, 100 Å) column equipped with a SeQuant® ZIC®-HILIC guard column (20 X 2.1 mm), both from Merck. Mobile phases consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The elution gradient used was as follows: 0.0, 95 % B; time range 0.0 to 0.5 mins, 95 % → 80 % B; time range 0.5 to 6.0 mins, 80 % → 40 % B; time range 6.0 to 8.0 mins, 40 % → 20 % B; time range 8.0 to 9.5 mins, 20 % B; time range 9.5 to 9.7 mins, 25 % → 95 % B and 9.7 to 12.2 mins, 95 % B (isocratic

column conditioning). The flow rate was set at 300 µl min-1, the injection volume was 3 µL and the column oven was maintained at 27 °C. Detection was performed in positive ionization mode. Quantifier and qualifier ions were as follows: Ornithine (133 → 70 / 133 → 116), Arginine (175 → 70 / 175 → 60), Citrulline (176 → 70 / 176 → 113), MNMA (189 → 70), SDMA (203 → 172 / 203 → 158), ADMA (203 → 46 / 203 → 158) and N4-Arginine (179 → 71). For additional confirmation, the ion ratios between the quantifier and qualifier ions for urea cycle amino acids (maximum deviation = 15 %) were calculated.

Data analysis

Data are represented as mean ± SEM. The statistical significance of differences between means was calculated by Student's t-test (inflammatory cytokines), or One or Two way ANOVA with Bonferroni's post-hoc test. Statistical analysis was performed with GraphPad Prism 7.0 software. Differences were considered to be statistically significant when $p<0.05$.

Results

In contrast to Arg1, Arg2 expression in mouse lung tissue is not upregulated in response to allergen challenge

We set out to test whether arginase 2 gene expression in mouse lungs could be regulated by allergen challenge. To achieve this, we analyzed the *Arg1* and *Arg2* expression in lung tissue of ovalbumin sensitized mice that were exposed to ovalbumin *in vivo*. As interleukin (IL)-13 is a key Th2-cytokine involved in airway inflammation in asthma, it was used as a control for Th2 inflammation (38). We observed a significant increase in *Arg1* and *IL-13* expression after allergen challenged compared to saline challenged controls, as expected ($p<0.05$) (Figure 1). Arginase 2 gene expression on the other hand, did not differ from control.

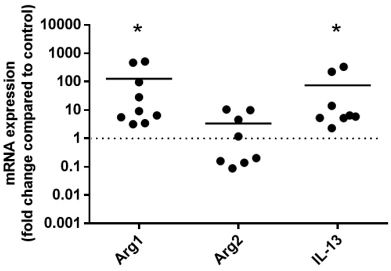

Figure 1: Effect of *in vivo* allergen challenge on arginase 1, arginase 2 and IL-13 gene expression in the lung of allergen sensitized mice compared to saline challenged animals. Dotted line represents gene expression of saline challenged mice. * $p<0.05$ compared to saline challenged animals. (n = 8-9 animals)

Arginase 2 deficiency affects mast cell-dependent airway narrowing

We further examined the role of the arginase 2 gene in allergic asthma using mice deficient in *Arg2* and an *ex vivo* lung slice model. Lung slices give the opportunity to test multiple conditions in the same animal, meaning that slices of the same animal can be used as a control (34, 39). Furthermore, the lung slice model allows for functional airway response studies in an intact tissue microenvironment (34, 39). Wild-type mice and *Arg2*^{KO} animals sensitized to ovalbumin were used for lung slice preparation. Dose-response curves were established to the nonselective muscarinic receptor agonist methacholine, and the mast cell activator compound 48/80 to test their effect on airway narrowing in wild-type and *Arg2*^{KO} mice. The results are shown in Figure 2. *Arg2*^{KO} animals did not differ in their responsiveness to methacholine compared to wild-type animals (Figure 2A). On the other hand, airway narrowing induced by compound 48/80, was slightly but significantly enhanced in *Arg2*^{KO} animals in comparison with wild-type animals ($p<0.05$; Figure 2B).

Inhibiting arginase 2 reduces cell number and size, but increases expression of mast cell receptors and mast cell mediator related enzymes

As we observed that arginase 2 gene-deficiency affects compound 48/80, but not methacholine induced airway narrowing, it seemed likely that arginase 2 plays a role in mast cell activation and not in smooth muscle contraction. In the lung, mast cells play an important role in allergic airway disease as they release among others pro-contractile and pro-inflammatory mediators in response to allergens (2). Human lung mast cells express high levels of arginase 2, whereas arginase 1 expression was not detected (30). Similarly, negligible expression of arginase 1 and high expression of arginase 2 can be found in the LUVA human mast cell line (Figure 3). Therefore, we used LUVA mast cells to further investigate the potential role of arginase 2 in mast cells. To inhibit arginase 2 activity in these cells, we used the specific, non-selective arginase inhibitor ABH (10 μM), since arginase 2 selective inhibitors are presently not available. The effect of arginase inhibition on cell number and size over time was determined.

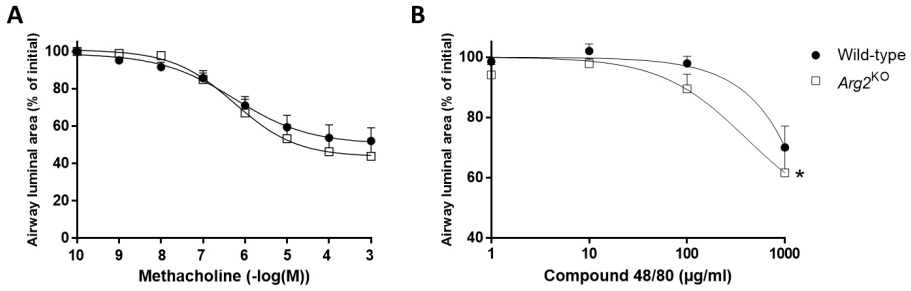

Figure 2: Methacholine (A) and compound 48/80 (B) induced airway narrowing in lung slices of Arg2KO and wild-type mice sensitized to allergen. Data represents mean ± SEM. * $p<0.05$, Two way ANOVA on complete curve (n = 6 animals)

As shown in Figure 4A, arginase 2 inhibition resulted in significant lower cell number over time compared to control ($p<0.01$ by Two way ANOVA), with a significant difference in cell number at 72h after treatment ($p<0.001$). Cell viability was not affected by ABH (Figure 4B). Furthermore, cell size was significantly affected by ABH after 48h and 72h of treatment ($p<0.05$; Figure 4C).
Reduced cell number, combined with no changes in cell viability and smaller cells may indicate a change in mast cell differentiation and proliferation. Therefore, we went on to determine the effect of arginase inhibition on expression levels of two genes encoding for differentiated mast cell receptors, *FCER1α* and *KIT*. LUVA cells were treated with ABH (10 µM) or left untreated. After 24h, mRNA was isolated and gene expression was measured by rtPCR. As shown in Figure 5A, gene expression of the mast cell differentiation marker *FCER1α* was significantly increased after ABH treatment compared to untreated control ($p<0.05$). *KIT* expression was not affected by arginase inhibition. The effect of arginase inhibition on gene expression of genes related to mast cell mediator production was also measured, for which the results are shown in Figure 5B. 24h treatment with ABH significantly increased the expression of *ALOX5*, *ALOX15*, *TPH2* and *PTGS2* ($p<0.05$), and *CMA1* ($p<0.01$) compared to control. Taken together these results indicate that treatment with ABH reduces LUVA cell proliferation and increases gene expression of receptors and enzymes involved in mast cell differentiation.

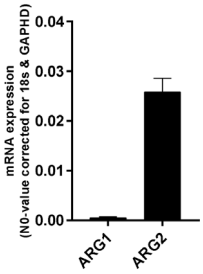

Figure 3: Arginase 1 and 2 gene expression in LUVA human mast cells. (n=4)

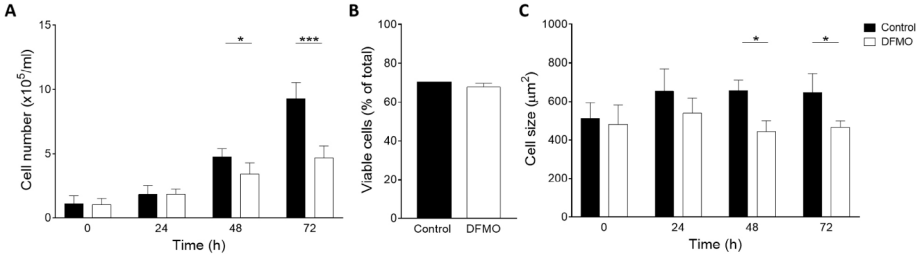

Figure 4: Effect of arginase inhibition with ABH (10 µM) on cell division (A), cell viability after 72h (B) and cell size (C) of LUVA mast cells. * $p<0.05$, *** $p<0.001$ compared to control. (n = 3-6)

Effect of arginase inhibition can be mimicked by inhibition of ODC

The next step was to unravel the mechanism behind the previously observed effects. Since arginase is involved in the conversion of L-arginine to L-ornithine, the first step was to measure whether arginase inhibition with ABH influenced intracellular arginine and L-ornithine levels in LUVA cells. As can be appreciated from Figure 7, treatment with ABH tended to increase intracellular arginine levels compared to untreated cells ($p=0.089$; Figure 6A), whereas a significant decrease in L-ornithine levels was observed ($p<0.001$; Figure 6B).

Downstream metabolic conversion of L-ornithine by ODC leads to the production of polyamines (putrescine, spermidine, spermine), which are known to be involved in many cellular processes, including proliferation and differentiation (40). We used the ODC inhibitor DL-α-difluoromethylornithine (DFMO; 5 mM) and hypothesized that this

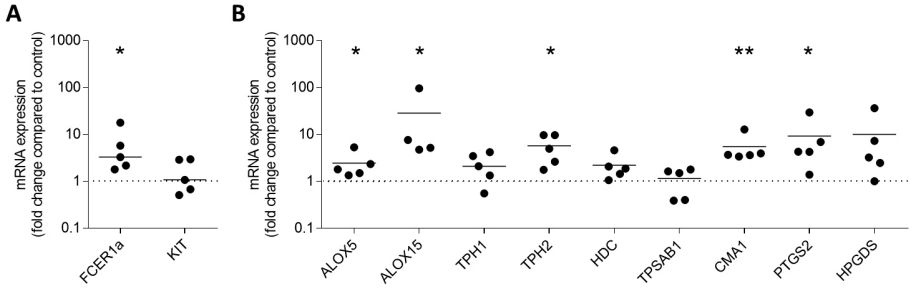

Figure 5: Effect of arginase inhibition with ABH (10 µM) on mRNA expression of mast cell receptor **(A)** and mast cell mediator enzymes **(B)** in LUVA mast cells. Dotted line represents gene expression of untreated control. * $p<0.05$, ** $p<0.01$ compared to control. (n = 4-5)

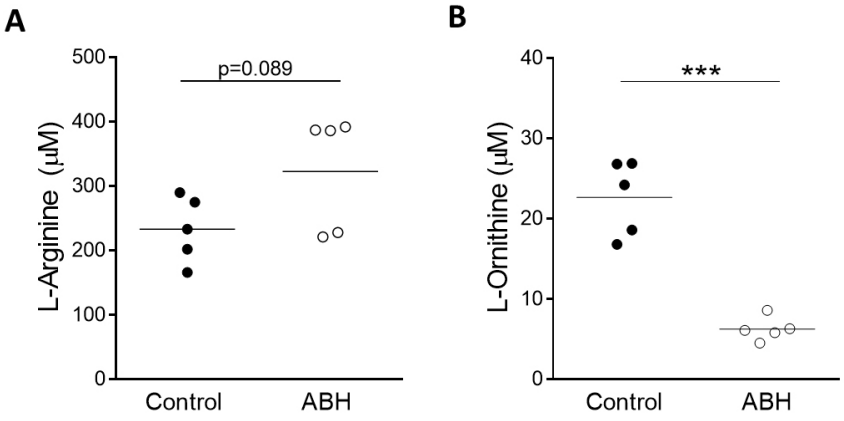

Figure 6: Effect of arginase inhibition with ABH (10 µM) on intracellular L-arginine **(A)** and L-ornithine **(B)** levels of LUVA mast cells. *** $p<0.001$ compared to control. (n = 5)

would mimic the effects that were observed after arginase inhibition. As shown in Figure 8, both mast cell proliferation (Figure 7A) and size (Figure 8C) were significantly reduced over time by treatment with DFMO compared to untreated cells ($p<0.001$ by Two way ANOVA), without affecting cell viability (Figure 7B). Cell numbers were significantly reduced after 48h ($p<0.05$) and 72h ($p<0.001$) of treatment. Cell size was also significantly reduced after 48h and 72h of ODC inhibition ($p<0.05$; Figure 7C).
Changes in gene expression of the mast cell receptors FcεR1 and c-KIT were also measured. As shown in Figure 8A, *FCER1α* expression was significantly upregulated after 24h treatment with DFMO ($p<0.01$). After 72h of treatment, *KIT* expression was significantly increased ($p<0.05$; Figure 8C). 24h treatment with DFMO did not affect gene expression of enzymes related to mast cell mediators (Figure 8B). However, after 72h *ALOX15* ($p<0.01$) and *PTGS2* ($p<0.05$) expression were significantly increased by DFMO treatment (Figure 8D). Taken together, these results indicate that ODC inhibition in LUVA cells indeed partially mimics the effect on mast cell proliferation and differentiation observed after arginase inhibition. Exceptions include *CMA1*, *ALOX5* and *TPH2*, which were not significantly affected by DFMO treatment.

ODC inhibition can be rescued by exogenous spermidine

In view of these results, we tried to rescue the effect of ODC inhibition with exogenous spermidine, one of the polyamines formed as a result of L-ornithine metabolism by ODC. LUVA cells were treated with DFMO (5 mM) and/or spermidine (100 µM) or left untreated for 96h. Over time, treatment with DFMO reduced cell number, which was completely rescued by spermidine ($p<0.001$ by Two way ANOVA; Figure 9). More specifically, treatment with DFMO reduced cell number at 72h and 96h compared to control ($p<0.001$). Combined treatment of DFMO and spermidine resulted in a lower increase in cell number compared to control after 72h ($p<0.01$). After 96h of treatment this difference was completely resolved, showing that spermidine is indeed able to reverse the effects induced by ODC inhibition.

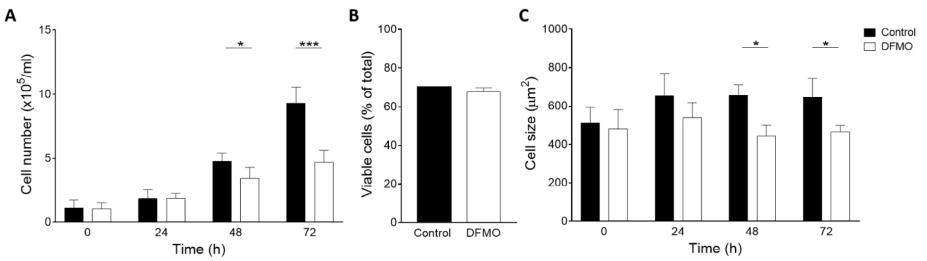

Figure 7: Effect of ODC inhibition with DFMO (5 mM) on cell proliferation **(A)**, cell viability **(B)** and cell size **(C)** of LUVA mast cells. * $p<0.05$, *** $p<0.001$ compared to control. (n = 3-6)

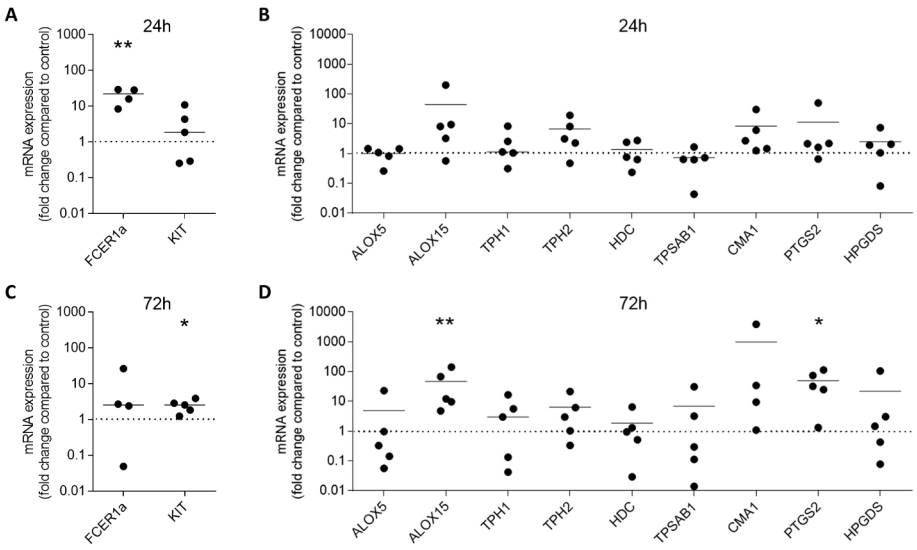

Figure 8: Effect of ODC inhibition with DFMO (5 mM) on mast cell receptor **(A, C)** and mediator **(B, D)** gene expression in LUVA mast cells after 24h **(A, B)** and 72h **(C, D)**. Dotted line represents gene expression of untreated control. * p<0.05, ** p<0.01 compared to control. (n = 4-5)

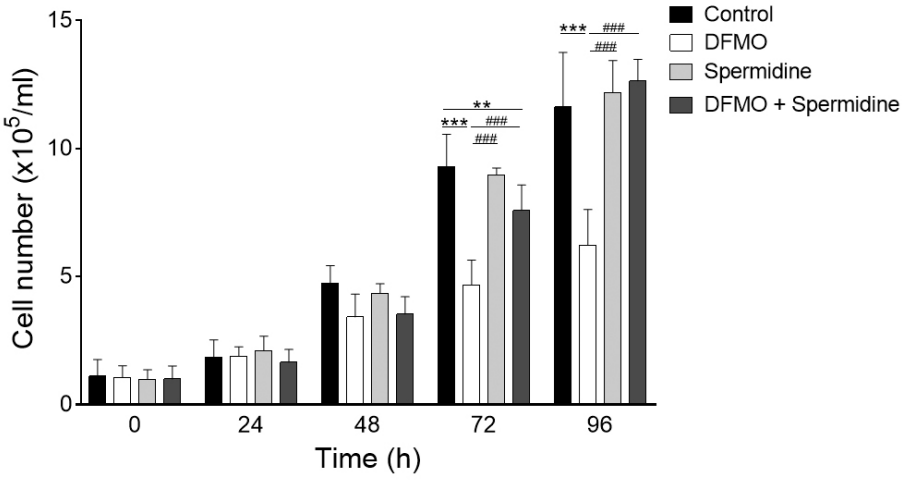

Figure 9: Effect of ODC inhibition with DFMO (5 mM) and/or exogenous spermidine (100 µM) on increases in cell number of LUVA mast cells. ** p<0.01, *** p<0.001 compared to control; ### p<0.001 compared to DFMO. (n = 6)

Discussion

The current study aimed to investigate the role of arginase 2 in mast cells. Using lung slices of mice deficient in *Arg2*, we found a small but significant protective effect of this gene on mast cell dependent airway narrowing. Further experiments using a human mast cell line showed that arginase 2 inhibition inhibits mast cell proliferation and reduces mast cell size, yet increases the gene expression of FcεR1 and enzymes involved in mast cell-mediator production, that arginase 2 activity promotes mast cell proliferation and inhibits differentiation towards a more differentiated phenotype. Furthermore, intracellular L-ornithine levels were decreased after arginase inhibition and inhibition of ODC led to comparable results on cell proliferation, cell size and gene expression as inhibition of arginase. Addition of exogenous spermidine was able to reverse the ODC inhibition effect on cell growth. Together this indicates that the protective role of arginase 2 on mast cell dependent airway narrowing is the result of the promotion of mast cell proliferation rather than differentiation, which is mediated via the regulation of L-ornithine metabolism

Interestingly, arginase 2 is the only detectable mast-cell expressed arginase subtype in both mast cell of asthma patients (41) and the main arginase isoenzyme expressed by LUVA human mast cells. We show that inhibition of this arginase activity in mast cells resulted in fewer, and smaller cells, that show increased expression of the mast cell differentiation marker IgE-receptor gene *FCER1α* and the mast cell mediator producing enzymes *ALOX5*, *ALOX15*, *TPH2*, *CMA1* and *PTGS2*. *FCER1α* encodes for the IgE-receptor, which initiates the allergic response (42). Mast cells of patients with mild or uncontrolled atopic asthma were found to have an increased expression of the FcεR1-receptors in comparison to healthy controls (43, 44). Moreover, *ALOX5*, *ALOX15* are *PTGS2* encode for enzymes involved in leukotriene and prostaglandin synthesis that are triggered by mast cell activation through the FcεR1-receptor (2), while *TPH2* and *CMA1* are involved in the production of serotonin and chymase, respectively, which are prestored in mast cell granules and are immediately released during mast cell degranulation (2). It is known that serotonin and histamine levels increase as mast cells mature (45, 46). The activity of the histamine producing enzyme histidine decarboxylase (HDC) was found to be slightly increased in response to DFMO in murine bone marrow-derived mast cells (47) and we observed a small albeit non-significant upregulation of *HDC* expression after arginase inhibition with ABH. Taken together, our results indicate that both arginase and ODC inhibition are able to induce a more differentiated mast cell phenotype, suggesting that mast cell-expressed arginase 2 plays a protective role, by preventing mast cell differentiation towards a more mature phenotype. This could explain the protective effect of arginase 2 on mast cell dependent airway narrowing observed in this study. Furthermore, we speculate that this protective role may even contribute to the reported protective effect of certain *ARG2* SNPs in the development, severity and treatment of asthma (19, 26, 27).

L-Arginine conversion by arginase provides an important source of L-ornithine for cells and L-ornithine metabolism by ODC is the vital first step in polyamine biosynthesis (10, 48). We found that inhibition of ODC activity in LUVA mast cells with DFMO produced similar effects as observed after inhibition of arginase using ABH. Both treatments resulted in fewer cells that were smaller, and in increased expression of mast cell differentiation markers. Furthermore, the effects of ODC inhibition could be rescued by addition of exogenous spermidine. Polyamines are polycations that interact with negatively charged molecules such as DNA, RNA and proteins and can modulate their biosynthesis (49). In this way, polyamines are able to affect numerous cellular processes, such as cell division and proliferation, cell survival, and DNA and protein synthesis (40), and are essential for cell growth and function (50-52). By promoting histone acetyltransferase activity and chromatin hyperacetylation, polyamines can stimulate the expression of genes involved in cell proliferation (53). Arginase has been suggested to play a role in cell proliferation via its contribution to polyamine biosynthesis in many cell types including endothelial cells (54), B- and T-cells (55) and blood cord-derived $CD34^{+}$-cells (56). Furthermore, depletion of polyamines by inhibiting arginase or ODC arrests cell growth (57-59), supporting our findings in LUVA mast cells. Polyamines are present in mast cell granules, are secreted in response to IgE and are important for granule structure (47). Several studies indicate an interaction between polyamines, mast cell granules and granule content (47, 60-62). In the granules of mast cells pre-made mediators are stored. Dependent of the species, these mediators include histamine, serotonin, chymases, tryptases and cytokines. We observed an upregulated gene expression of *TPH2* and *CMA1* after arginase inhibition. The *THP2* and *CMA1* enzymes are involved in the production of serotonin and chymase, respectively (63, 64), that are stored in granules. In rat peritoneal mast cells, it was observed that immediately following secretion, cell volume was markedly decreased, with cells taking over a month to return to their original volume (46).Treatment of mouse bone marrow-derived mast cells with DFMO resulted in morphological changes in granules (47, 60). We speculate that these morphological changes might also affect mast cell volume, thereby offering a possible explanation why we observed a decrease in cell size after polyamine depletion by ABH or DFMO.

We also used mouse precision-cut lung slices of mice deficient in *Arg2* to examine the, still largely unknown, functional role of arginase 2 in mast cells. In the lung slice model, functional airway responses can be studied in an intact lung microenvironment (34, 39), thus providing a unique approach to investigate integrated molecular, cellular and mechanic responses in the lung. In our study, $Arg2^{KO}$ did not affect airway narrowing by methacholine, which is a direct stimulus of the airway smooth muscle. This is consistent with findings in asthmatic patients that show that arginase 2 expression and arginase activity were not related to airway reactivity to methacholine (19). It is also an important experimental control for our experiments, as it shows the baseline dynamics of airway narrowing not altered in the $Arg2^{KO}$ mice. In contrast, compound 48/80-induced airway narrowing was increased by $Arg2^{KO}$. Compound 48/80 is an indirect stimulus of airway

smooth muscle contraction by causing both activation and degranulation of mast cells in a non-IgE dependent manner (65).

Both *ARG1* and *ARG2* gene variants have been linked to asthma susceptibility and severity (16-18). The *ARG2* gene is located on the asthma linkage region of chromosome 14q24 (66), which suggests its importance in asthma pathology. Until recently high arginase 2 protein and gene expression in asthmatic airways were assumed to be detrimental. However, Xu et al showed that *Arg2*^{KO} mice had increased Th2 inflammation than wild-type mice and *ARG2* overexpression in bronchial epithelial cells can suppress *HIF* and *STAT6* activation, thereby dampening IL-13 mediated signaling (26). Furthermore, *Arg2*^{KO} mice have increased eosinophilic and neutrophilic airway inflammation and increased angiogenic remodeling in response to allergen exposure (27). These findings were all found to be related to interactions between arginase 2 and inducible NOS (iNOS) via TCA cycle activity in mitochondria and intracellular L-arginine metabolism, independently of substrate competition (19, 26, 27). It is suggested, that L-ornithine generated by arginase 2 in mitochondria acts, via ornithine aminotransferase as a precursor for glutamate. Transamination of glutamate via ornithine oxaloacetate, will give rise to aspartate, which via argininosuccinate synthetase and argininosuccinate lyase serves as a source for the formation of L-arginine for NO generation by iNOS (26). Here, we report that arginase 2 also affects mast cell proliferation and maturation by influencing L-ornithine metabolism via ODC. It might well be possible that these pathways overlap, as L-ornithine has also been shown to play a function as a precursor for glutamate in the TCA cycle, however this has not been studied so far. Nevertheless, L-ornithine metabolism has already been proven to be related to allergic asthma, as increased L-ornithine-derived polyamines cause airway hyperresponsiveness in a mouse model of asthma (67). Furthermore, increased putrescine levels have been observed in mouse lungs after allergen challenge (13) and elevated levels of putrescine, spermidine and spermine could be detected in serum of asthmatic patients (68). Thus, arginase 2 is not only involved in protection from allergic asthma via its in role in mitochondrial metabolism that interacts with iNOS, but in certain cell types, such as mast cells, probably also by influencing L-ornithine metabolism.

In conclusion, our study shows that arginase 2 has a protective effect on mast cell differentiation towards a more mature phenotype, which might contribute to decreased mast cell dependent airway narrowing. This is mediated by L-ornithine metabolism via ODC. Our findings broaden the understanding of the role of arginase 2 in allergic airway inflammation and show for the first time a role for arginase 2 in mast cells.

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References

1. Masoli M, Fabian D, Holt S, Beasley R, Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy. 2004;59(5):469-78.
2. Reuter S, Stassen M, Taube C. Mast cells in allergic asthma and beyond. Yonsei Med J. 2010;51(6):797-807.
3. Liu MC, Hubbard WC, Proud D, Stealey BA, Galli SJ, Kagey-Sobotka A, et al. Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics: cellular, mediator, and permeability changes. Am Rev Respir Dis. 1991;144(1):51-8.
4. Murray JJ, Tonnel AB, Brash AR, Roberts LJ,2nd, Gosset P, Workman R, et al. Prostaglandin D2 is released during acute allergic bronchospasm in man. Trans Assoc Am Physicians. 1985;98:275-80.
5. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. Am J Respir Crit Care Med. 2000;161(5):1720-45.
6. Martin JG, Duguet A, Eidelman DH. The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease. Eur Respir J. 2000;16(2):349-54.
7. Maarsingh H, Leusink J, Bos IST, Zaagsma J, Meurs H. Arginase strongly impairs neuronal nitric oxide-mediated airway smooth muscle relaxation in allergic asthma. Respir Res. 2006;7(1):6.
8. Maarsingh H, Bossenga BE, Bos IS, Volders HH, Zaagsma J, Meurs H. L-arginine deficiency causes airway hyperresponsiveness after the late asthmatic reaction. Eur Respir J. 2009;34(1):191-9.
9. Meurs H, McKay S, Maarsingh H, Hamer MA, Macic L, Molendijk N, et al. Increased arginase activity underlies allergen-induced deficiency of cNOS-derived nitric oxide and airway hyperresponsiveness. Br J Pharmacol. 2002;136(3):391-8.
10. Wu G, Morris SM. Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(1):1-17.
11. Jenkinson CP, Grody WW, Cederbaum SD. Comparative properties of arginases. Comp Biochem Physiol B, Biochem Mol Biol. 1996;114(1):107-32.
12. Maarsingh H, Zuidhof AB, Bos IST, van Duin M, Boucher J, Zaagsma J, et al. Arginase inhibition protects against allergen-induced airway obstruction, hyperresponsiveness, and inflammation. Am J Respir Crit Care Med. 2008;178(6):565-73.
13. Zimmermann N, King NE, Laporte J, Yang M, Mishra A, Pope SM, et al. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. J Clin Invest. 2003;111(12):1863-74.
14. North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. Am J Physiol Lung Cell Mol Physiol. 2009;296(6):911.
15. Maarsingh H, Dekkers BG, Zuidhof AB, Bos IS, Menzen MH, Klein T, et al. Increased arginase activity contributes to airway remodelling in chronic allergic asthma. Eur Respir J. 2011;38(2):318-28.
16. Vonk JM, Postma DS, Maarsingh H, Bruinenberg M, Koppelman GH, Meurs H. Arginase 1 and arginase 2 variations associate with asthma, asthma severity and beta2 agonist and steroid response. Pharmacogenet Genomics. 2010;20(3):179-86.

17. Salam, Muhammad T., MS, MD, Islam, Talat, MBBS, PhD, Gauderman WJ, PhD, Gilliland, Frank D., MD, PhD. Roles of arginase variants, atopy, and ozone in childhood asthma. J Allergy Clin Immunol. 2009;123(3):596,602.e8.
18. Li H, Romieu I, Sienra-Monge J, Ramirez-Aguilar M, Estela del Rio-Navarro B, Kistner EO, et al. Genetic polymorphisms in arginase I and II and childhood asthma and atopy. J Allergy Clin Immunol. 2006;117(1):119-26.
19. Xu W, Comhair SA, Janocha AJ, Lara A, Mavrakis LA, Bennett CD, et al. Arginine metabolic endotypes related to asthma severity. PloS one. 2017;12(8):e0183066.
20. Morris CR, Poljakovic M, Lavrisha L, Machado L, Kuypers FA, Morris Jr SM. Decreased arginine bioavailability and increased serum arginase activity in asthma. Am J Respir Crit Care Med. 2004;170(2):148-53.
21. Meurs H, Zaagsma J, Maarsingh H, van Duin M. Recent Patents in Allergy/Immunology: Use of arginase inhibitors in the treatment of asthma and allergic rhinitis. Allergy. 2019;74(6):1206-8.
22. Barron L, Smith AM, El Kasmi KC, Qualls JE, Huang X, Cheever A, et al. Role of arginase 1 from myeloid cells in th2-dominated lung inflammation. PloS one. 2013;8(4):e61961.
23. Cloots RHE, Sankaranarayanan S, de Theije CC, Poynter ME, Terwindt E, van Dijk P, et al. Ablation of Arg1 in hematopoietic cells improves respiratory function of lung parenchyma, but not that of larger airways or inflammation in asthmatic mice. Am J Physiol Lung Cell Mol Physiol. 2013;305(5):364.
24. Cloots RH, Sankaranarayanan S, Poynter ME, Terwindt E, van Dijk P, Lamers WH, et al. Arginase 1 deletion in myeloid cells affects the inflammatory response in allergic asthma, but not lung mechanics, in female mice. BMC Pulm Med. 2017;17(1):158.
25. Aristoteles, Luciana R. C. R. B., Righetti RF, Pinheiro NM, Franco RB, Starling CM, da Silva, Julie C. P., et al. Modulation of the oscillatory mechanics of lung tissue and the oxidative stress response induced by arginase inhibition in a chronic allergic inflammation model. BMC Pulm Med. 2013;13:52.
26. Xu W, Ghosh S, Comhair SAA, Asosingh K, Janocha AJ, Mavrakis DA, et al. Increased mitochondrial arginine metabolism supports bioenergetics in asthma. J Clin Invest. 2016;126(7):2465-81.
27. Asosingh K, Lauruschkat CD, Alemagno M, Frimel M, Wanner N, Weiss K, et al. Arginine metabolic control of airway inflammation. JCI insight. 2020:5(2):e127801.
28. Yamamoto M, Tochino Y, Chibana K, Trudeau JB, Holguin F, Wenzel SE. Nitric oxide and related enzymes in asthma: relation to severity, enzyme function and inflammation. Clin Exp Allergy. 2012;42(5):760-8.
29. Lonsdale J, Thomas J, Salvatore M, Phillips R, Lo E, Shad S, et al. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013;45(6):580-5.
30. Adams TS, Schupp JC, Poli S, Ayaub EA, Neumark N, Ahangari F, et al. Single Cell RNA-seq reveals ectopic and aberrant lung resident cell populations in Idiopathic Pulmonary Fibrosis. BioRxiv. 2019:759902.
31. Shi O, Morris SM, Zoghbi H, Porter CW, O'Brien WE. Generation of a mouse model for arginase II deficiency by targeted disruption of the arginase II gene. Mol Cell Biol. 2001;21(3):811-3.
32. Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, van der Strate, Barry WA, et al. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. Am J Resp Cell Mol. 2004;30(6):880-5.

33. Dragunas G, Woest ME, Nijboer S, Bos ST, van Asselt J, de Groot AP, et al. Cholinergic neuroplasticity in asthma driven by TrkB signaling. FASEB J. 2020;34:7703-17
34. Wu X, van Dijk EM, Bos IST, Kistemaker LEM, Gosens R. Mouse Lung Tissue Slice Culture. Methods Mol Biol. 2019;1940:297-311.
35. Van Dijk EM, Culha S, Menzen MH, Bidan CM, Gosens R. Elastase-Induced Parenchymal Disruption and Airway Hyper Responsiveness in Mouse Precision Cut Lung Slices: Toward an *Ex vivo* COPD Model. Front Physiol. 2017;7:657.
36. Xu L, Hilliard B, Carmody RJ, Tsabary G, Shin H, Christianson DW, et al. Arginase and autoimmune inflammation in the central nervous system. Immunology. 2003;110(1):141-8.
37. Rigobello MP, Scutari G, Boscolo R, Bindoli A. Induction of mitochondrial permeability transition by auranofin, a Gold(I)-phosphine derivative. Br J Pharmacol. 2002;136(8):1162-8.
38. Ingram JL, Kraft M. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. J Allergy Clin Immunol. 2012;130(4):829-42.
39. Martin C, Uhlig S, Ullrich V. Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. Eur Respir J. 1996;9(12):2479-87.
40. Lenis YY, Elmetwally MA, Maldonado-Estrada JG, Bazer FW. Physiological importance of polyamines. Zygote. 2017;25(3):244-55.
41. Braga FAV, Kar G, Berg M, Carpaij OA, Polanski K, Simon LM, et al. A cellular census of human lungs identifies novel cell states in health and in asthma. Nat Med. 2019;25(7):1153-63.
42. Turner H, Kinet J. Signalling through the high-affinity IgE receptor FcεRI. Nature. 1999;402(6760):24-30.
43. Andersson CK, Tufvesson E, Aronsson D, Bergqvist A, Mori M, Bjermer L, et al. Alveolar mast cells shift to an FcεRI-expressing phenotype in mild atopic asthma: a novel feature in allergic asthma pathology. Allergy. 2011;66(12):1590-7.
44. Andersson C, Bergqvist A, Mori M, Mauad T, Bjermer L, Erjefält J. Mast cell-associated alveolar inflammation in patients with atopic uncontrolled asthma. J Allergy Clin Immunol. 2011;127(4):123.
45. Krüger PG, Lagunoff D. Effect of age on mast cell granules. Int Arch Allergy Immunol. 1981;65(3):291-9.
46. Hammel I, Lagunoff D, Krüger P-. Recovery of rat mast cells after secretion: A morphometric study. Exp Cell Res. 1989;184(2):518-23.
47. García-Faroldi G, Rodríguez CE, Urdiales JL, Pérez-Pomares JM, Dávila JC, Pejler G, et al. Polyamines Are Present in Mast Cell Secretory Granules and Are Important for Granule Homeostasis. PLoS One. 2010;5(11):e15071.
48. Morris SM,Jr. Arginine Metabolism Revisited. J Nutr. 2016;146(12):2579S-86S.
49. Igarashi K, Kashiwagi K. Polyamines: mysterious modulators of cellular functions. Biochem Biophys Res Commun. 2000;271(3):559-64.
50. Heby O. Role of polyamines in the control of cell proliferation and differentiation. Differentiation. 1981;19(1-3):1-20.
51. Marton LJ, Pegg AE. Polyamines as targets for therapeutic intervention. Annu Rev Pharmacol Toxicol. 1995;35(1):55-91.
52. Wallace HM. The physiological role of the polyamines. Eur J Clin Invest. 2000;30(1):1.
53. Hobbs CA, Gilmour SK. High levels of intracellular polyamines promote histone acetyltransferase activity resulting in chromatin hyperacetylation. J cell Biochem. 2000;77(3):345-60.
54. Li H, Meininger CJ, Kelly KA, Hawker Jr JR, Morris Jr SM, Wu G. Activities of arginase I and II are limiting for endothelial cell proliferation. Am J Physiol-Reg I. 2002;282(1):R64-9.

55. Hesterberg RS, Cleveland JL, Epling-Burnette PK. Role of polyamines in immune cell functions. Med Sci. 2018;6(1):22.
56. Maeda T, Wakasawa T, Shima Y, Tsuboi I, Aizawa S, Tamai I. Role of polyamines derived from arginine in differentiation and proliferation of human blood cells. Biol Pharm Bull. 2006;29(2):234-9.
57. Morrison RF, Seidel ER. Vascular endothelial cell proliferation: regulation of cellular polyamines. Cardiovasc Res. 1995;29(6):841-7.
58. Takigawa M, Enomoto M, Nishida Y, Pan H, Kinoshita A, Suzuki F. Tumor angiogenesis and polyamines: Î±-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibits B16 melanoma-induced angiogenesis in ovo and the proliferation of vascular endothelial cells *in vitro*. Cancer Res. 1990;50(13):4131-8.
59. Wei LH, Wu G, Morris SM, Ignarro LJ. Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation. PNAS. 2001;98(16):9260-4.
60. Nishimura K, Okamoto M, Shibue R, Mizuta T, Shibayama T, Yoshino T, et al. KLF4 is required for suppression of histamine synthesis by polyamines during bone marrow-derived mast cell differentiation. PLoS One. 2020 -2-26;15(2).
61. Vliagoftis H, Mak L, Boucher W, Theoharides TC. Dual effect of spermine on mast cell secretion exhibits different calcium and temperature requirements. International Journal of Immunopharmacology. 1999;21(9):547-59.
62. Acosta-Andrade C, Lambertos A, Urdiales JL, Sánchez-Jiménez F, Peñafiel R, Fajardo I. A novel role for antizyme inhibitor 2 as a regulator of serotonin and histamine biosynthesis and content in mouse mast cells. Amino Acids. 2016;48(10):2411-21.
63. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annu Rev Med. 2009;60:355-66.
64. Caughey GH, Schaumberg TH, Zerweck EH, Butterfield JH, Hanson RD, Silverman GA, et al. The Human Mast Cell Chymase Gene (CMA1): Mapping to the Cathepsin G/Granzyme Gene Cluster and Lineage-Restricted Expression. Genomics. 1993;15(3):614-20.
65. Schemann M, Kugler EM, Buhner S, Eastwood C, Donovan J, Jiang W, et al. The Mast Cell Degranulator Compound 48/80 Directly Activates Neurons. PLOS ONE. 2012;7(12):e52104.
66. Hakonarson H, Bjornsdottir US, Halapi E, Palsson S, Adalsteinsdottir E, Gislason D, et al. A Major Susceptibility Gene for Asthma Maps to Chromosome 14q24. Am J Hum Genet. 2002;71(3):483-91.
67. North ML, Grasemann H, Khanna N, Inman MD, Gauvreau GM, Scott JA. Increased ornithine-derived polyamines cause airway hyperresponsiveness in a mouse model of asthma. Am J Respir Cell Mol Biol. 2013;48(6):694-702.
68. Kurosawa M, Shimizu Y, Tsukagoshi H, Ueki M. Elevated levels of peripheralâ€ blood, naturally occurring aliphatic polyamines in bronchial asthmatic patients with active symptoms. Allergy. 1992;47(6):638-43.